High-level resistance to isoniazid and ethionamide in multidrug-resistant Mycobacterium tuberculosis of the Lisboa family is associated with inhA double mutations

Machado, Diana ; Perdigão, João ; Ramos, Jorge ; Couto, Isabel ; Portugal, Isabel ; Ritter, Claudia ; Boettger, Erik C ; Viveiros, Miguel

Abstract: OBJECTIVES: The purpose of this study was to determine the levels of isoniazid and ethionamide resistance and to identify associated mutations in endemic multidrug-resistant (MDR) strains of Mycobacterium tuberculosis from the Lisbon metropolitan area, Portugal. METHODS: Seventeen clinical MDR tuberculosis (TB) strains were characterized by standard and semi-quantitative drug susceptibility testing to assess the level of isoniazid and ethionamide resistance. The genes katG, inhA, ethA and ndh were screened for mutations. All strains were genotyped by 24 loci mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) analysis. RESULTS: All strains showed high-level resistance to both isoniazid (>1 mg/L) and ethionamide (>25 mg/L). MIRU-VNTR typing revealed the presence of two main clusters, Lisboa3 and Q1, in 16/17 strains, all of which showed the C-15T mutation in the promoter region of the inhA gene. The 16 strains belong to the Latino-American-Mediterranean (LAM) genotype and the other strain belongs to the Beijing genotype. Sequencing of the inhA open reading frame revealed that the 16 strains also had mutations in the structural region of the gene, leading to the S94A substitution in 9 strains and the I194T substitution in 7 strains. CONCLUSIONS: The results reveal that the presence of a mutation in the inhA regulatory region together with a mutation in the inhA coding region can lead to the development of high-level isoniazid resistance and cross-resistance to ethionamide among the MDR-TB strains circulating in Lisbon. This mutational pattern also hints to a possible involvement of strain-specific factors that could be a feature of the Portuguese MDR-TB strains where the LAM family is the major circulating genotype.

DOI: https://doi.org/10.1093/jac/dkt090

J Antimicrob Chemother 2013; **68**: 1728–1732
doi:10.1093/jac/dkt090 Advance Access publication 28 March 2013

Journal of Antimicrobial Chemotherapy

High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations

Diana Machado[1], João Perdigão[2], Jorge Ramos[1], Isabel Couto[1,3], Isabel Portugal[2], Claudia Ritter[4], Erik C. Boettger[4] and Miguel Viveiros[1]*

[1]Grupo de Micobactérias, Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL), Rua da Junqueira 100, 1349-008 Lisboa, Portugal; [2]Centro de Patogénese Molecular/URIA, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal; [3]Centro de Recursos Microbiológicos (CREM), Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal; [4]Institut für Medizinische Mikrobiologie, Nationales Zentrum für Mykobakterien, Universität Zürich, Zürich, Switzerland

*Corresponding author. Tel: +351-213652653; Fax: +351-213632105; E-mail: mviveiros@ihmt.unl.pt

Received 7 October 2012; returned 25 November 2012; revised 17 February 2013; accepted 19 February 2013

Objectives: The purpose of this study was to determine the levels of isoniazid and ethionamide resistance and to identify associated mutations in endemic multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* from the Lisbon metropolitan area, Portugal.

Methods: Seventeen clinical MDR tuberculosis (TB) strains were characterized by standard and semi-quantitative drug susceptibility testing to assess the level of isoniazid and ethionamide resistance. The genes *katG*, *inhA*, *ethA* and *ndh* were screened for mutations. All strains were genotyped by 24 loci mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU-VNTR) analysis.

Results: All strains showed high-level resistance to both isoniazid (>1 mg/L) and ethionamide (>25 mg/L). MIRU-VNTR typing revealed the presence of two main clusters, Lisboa3 and Q1, in 16/17 strains, all of which showed the C−15T mutation in the promoter region of the *inhA* gene. The 16 strains belong to the Latino-American-Mediterranean (LAM) genotype and the other strain belongs to the Beijing genotype. Sequencing of the *inhA* open reading frame revealed that the 16 strains also had mutations in the structural region of the gene, leading to the S94A substitution in 9 strains and the I194T substitution in 7 strains.

Conclusions: The results reveal that the presence of a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region can lead to the development of high-level isoniazid resistance and cross-resistance to ethionamide among the MDR-TB strains circulating in Lisbon. This mutational pattern also hints to a possible involvement of strain-specific factors that could be a feature of the Portuguese MDR-TB strains where the LAM family is the major circulating genotype.

Keywords: tuberculosis, MDR-TB, resistance levels, LAM family

Introduction

Isoniazid is one of the most effective drugs for the treatment of tuberculosis (TB). Ethionamide is a second-line drug used in the treatment of multidrug-resistant (MDR) TB. Both compounds are pro-drugs that need activation by different enzymes but share common pathways, which can lead to cross-resistance. The majority of the mutations responsible for high-level isoniazid resistance in *Mycobacterium tuberculosis* are found in the *katG* gene.[1] The second most commonly found mutations are observed in the promoter of *inhA*.[1] These mutations increase InhA expression and confer low-level resistance to isoniazid ($0.1 < \text{MIC} < 1.0$ mg/L).[2] Mutations in the *inhA* promoter also confer resistance to ethionamide, whose activity against *M. tuberculosis* depends on activation by EthA.[3] In addition to *inhA* promoter mutations, decreased susceptibility to isoniazid and ethionamide is associated with mutations in the structural region of *inhA* that decrease the affinity to the drug–NAD adduct.[2]

In Portugal, the incidence rate of TB is high and the prevalence of MDR-TB and extensively drug-resistant (XDR) TB in the Lisbon region is a cause of major concern.[4] More than 90% of these strains show high-level isoniazid resistance associated

with the C−15T *inhA* promoter mutation. This genotype was also related with the high prevalence of ethionamide resistance.[5] To address the apparent incomprehensiveness between the level of isoniazid and ethionamide resistance in these strains and their mutation profile, we analysed the level of resistance towards isoniazid and ethionamide and identified associated mutations in MDR-TB strains isolated during 2009–11 at the Mycobacteriology Laboratory of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa.

Materials and methods

Strains

Seventeen clinical strains of *M. tuberculosis* were included in the study (see Table 1). The isolates were obtained from different patients with active TB from hospitals in the Lisbon region and correspond to all MDR-TB strains received in our laboratory during 2009–11 for identification and first-line susceptibility testing. The selection criterion for this study was only MDR strains. All isolates were identified using the AccuProbe MTBC test (GenProbe Inc., San Diego, CA, USA) according to the manufacturer's instructions. The *M. tuberculosis* reference strain H37Rv ATCC27294^T was included as the control.

Susceptibility testing

First-line susceptibility testing was carried out with the BACTEC™ MGIT™ 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) for streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide, according to the manufacturer's instructions. Semi-quantitative drug susceptibility testing of isoniazid and ethionamide was conducted using the MGIT 960 system and EpiCenter V5.80A software equipped with the TB eXIST module (Becton Dickinson), as previously described.[6,7]

Detection of mutations associated with resistance

Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The most common mutations in the structural *katG* gene and in the *inhA* regulatory region were investigated using Genotype MTBDR*plus* (Hain Lifescience GmbH, Germany) according to the manufacturer's instructions. Nucleic acid analysis of the complete *inhA*, *katG* and *ethA* genes and an internal fragment of the *ndh* gene (nt 560–931) (for a list of the primers and conditions see Table S1, available as Supplementary data at *JAC* Online) was performed by DNA sequencing.

Strain typing

Mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU-VNTR) genotyping was performed by multiplex PCR amplification of 24 loci, as described by Supply *et al.*[8] The genotype of these strains was analysed using the MIRU-VNTR*plus* web application[9] and the SITVITWEB database[10] when relevant.

Results

The quantitative isoniazid resistance levels were determined for the 17 MDR-TB isolates and the results are summarized in Table 1. All isolates display isoniazid resistance levels >1 mg/L. According to the Genotype MTBDR*plus* assay, 16 of the 17 strains contain a mutation the *inhA* promoter region (C−15T); one strain (MTB12) has a mutation in the *katG* gene (S315T). The entire *katG* gene was sequenced for a selected group of strains (MTB2, 4, 5, 9, 10 and 13–17), but no mutation was found. The C−15T mutation is described to confer cross-resistance to ethionamide. All isolates show high-level ethionamide resistance (>25 mg/L) (Table 1). Sequencing of the *inhA* open reading frame (ORF) revealed that all strains in addition to the C−15T mutation in the *inhA* promoter harbour point mutations in the *inhA* structural gene (Table 1). Nine of these isolates carry the S94A mutation. In the remaining seven isolates, we detected the I194T mutation. The single isolate with a mutation in *katG*, in addition carries a mutation previously described in *ethA*, S266R.[11] No mutation was found in the region of the *ndh* gene analysed.

Analysis of the 17 strains by MIRU-VNTR typing revealed that 16/17 strains represent two clusters, cluster Lisboa3 and cluster Q1 (Figure 1). These 16 strains can be assigned to the Latino-American-Mediterranean (LAM) genotype and the remaining 1 to the Beijing genotype. Cluster Lisboa3 comprised the nine strains with the C−15T/S94A mutations and the seven strains with the C−15T/I194T substitutions were clustered in Q1 (Figure 1).

Discussion

In the present study, we determined the levels of resistance to isoniazid and ethionamide for all MDR-TB strains isolated from 2009 to 2011 in our laboratory and we searched for mutations in target genes associated with resistance to isoniazid and ethionamide. We found that 16 strains have cumulative mutations in the *inhA* gene: the C−15T mutation in the regulatory region coupled with mutations in the structural region of the gene, leading to the S94A and I194T substitutions. The presence of the mutation C−15T has been reported to confer low-level resistance to isoniazid and cross-resistance to ethionamide.[1] The mutations S94A[2] and I194T[12,13] have also been reported to cause isoniazid resistance and both were associated with low-level resistance to isoniazid.[11,13] However, there are few reports on the combinations C−15T/S94A[3,11] and C−15T/I194T.[12] The double mutation C−15T/S94A has been reported to confer low-level resistance,[11] while the C−15T/I194T combination was identified in one isolate with high-level resistance to isoniazid.[12] All 16 strains with a double mutation studied in our work exhibit high-level resistance to both isoniazid and ethionamide. In an attempt to compare the level of resistance of these strains carrying double mutations with the resistance level of strains carrying only the C−15T mutation, we searched for additional isoniazid-resistant strains in our laboratory isolated during the same period. This C−15T mutation alone was found among non-MDR strains and was correlated with low-level resistance to isoniazid (data not shown).

MIRU-VNTR typing of the strains showed the presence of two main clusters, Lisboa3 and Q1, indicating that the dissemination of these strains is clonal. However, the mutational profile related to drug resistance indicates that the acquisition of drug resistance-associated mutations is non-clonal (data not shown), except for isoniazid and rifampicin. Thus, resistance to isoniazid has been transmitted clonally, as all strains share the same C−15T mutation and the S94A/I194T mutations were acquired subsequent to the C−15T mutation. Sixteen out of 17

Table 1. Quantitative drug susceptibility profile of isoniazid and ethionamide and associated mutations in the 17 *M. tuberculosis* isolates

Strain	Standard DST profile	INH qDST (mg/L)					ETH qDST (mg/L)			Gene mutations				
		0.1	0.4	1	3	10	5	10	25	*inhA*prom	*inhA* ORF	*katG*	*ethA*	*ndh*
H37Rv	INH^S, RIF^S, STR^S, ETB^S, PZA^S	S	S	S	S	S	S	S	S	none	none	none	none	none
MTB1	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C−15T	**S94A**	none	**none**	none
MTB2	INH^R, RIF^R, STR^R, ETB^S, PZA^S	R	R	R	S	S	R	R	R	C−15T	**S94A**	**none**	**none**	none
MTB3	INH^R, RIF^R, STR^R, ETB^S, PZA^S	R	R	R	S	S	R	R	R	C−15T	**S94A**	none	**none**	none
MTB4	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C−15T	**S94A**	**none**	**none**	none
MTB5	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	**none**	**none**	none
MTB6	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	none	**none**	none
MTB7	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	none	**none**	none
MTB8	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C−15T	**S94A**	none	**none**	none
MTB9	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	R	R	R	R	R	C−15T	**S94A**	**none**	**none**	none
MTB10	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	**none**	**none**	none
MTB11	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**S94A**	none	**none**	none
MTB12	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	R	R	R	R	R	none	**none**	S315T	**S266R**	none
MTB13	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	I	S	R	R	R	C−15T	**I194T**	**none**	**none**	none
MTB14	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**S94A**	**none**	**none**	none
MTB15	INH^R, RIF^R, STR^R, ETB^R, PZA^S	R	R	R	S	S	R	R	R	C−15T	**S94A**	**none**	**none**	none
MTB16	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	**none**	**none**	none
MTB17	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C−15T	**I194T**	**none**	**none**	none

MTB, *M. tuberculosis*; DST, drug susceptibility testing; qDST, quantitative drug susceptibility testing; prom, promoter; INH, isoniazid; ETH, ethionamide; RIF, rifampicin; STR, streptomycin; ETB, ethambutol; PZA, pyrazinamide; R, resistant; I, intermediate; S, susceptible.
Results in bold indicate that the entire gene was sequenced.

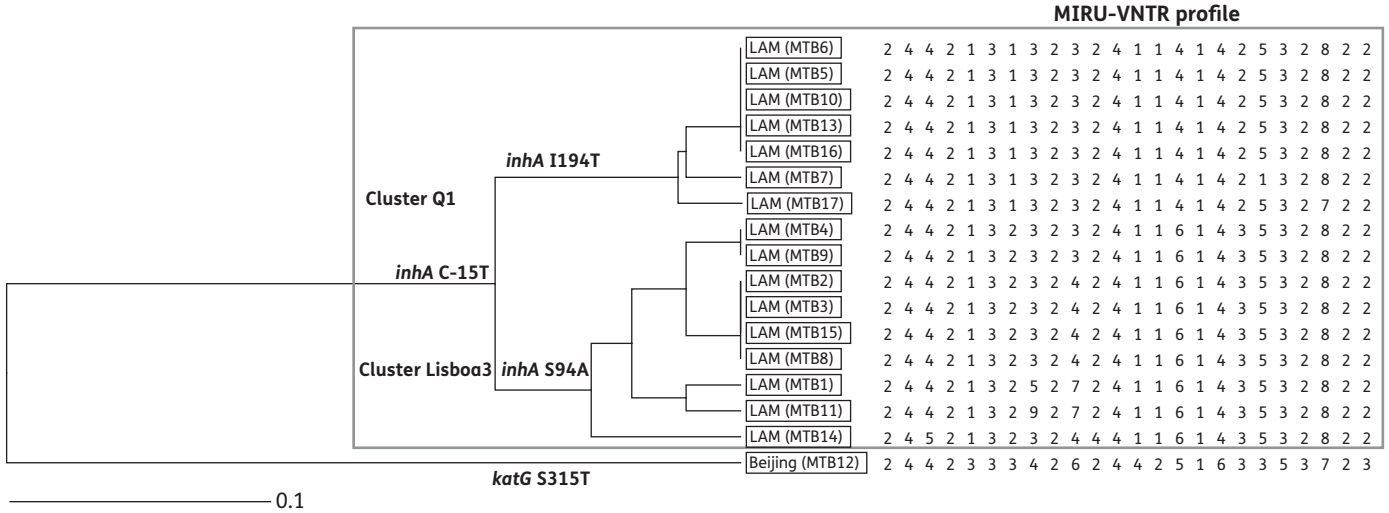

Figure 1. Cladogram based on 24 loci MIRU-VNTR of the 17 MDR *M. tuberculosis* isolates from Lisbon, Portugal. The grey box highlights clusters Q1 and Lisboa3 (both belonging to the LAM genotype). From left to right are shown lineage, strain number and MIRU-VNTR profile. The linkage distance scale is indicated at the bottom. LAM, Latino-American-Mediterranean; MTB, *M. tuberculosis*.

strains were found to belong to the LAM genotype, previously shown to be the main genotype circulating in Portugal.[5,14] Fenner *et al*.[15] recently proposed that the genetic background of a strain may influence the level of resistance to isoniazid conveyed by particular drug resistance-conferring mutations. Our results suggest that these two mutations, when combined with the C−15T mutation, can act synergistically to confer high-level resistance in this particular group of strains. All non-MDR

strains evaluated during the period of this study, with low-level resistance to isoniazid, belong to the LAM genotype, with only one belonging to Lisboa3 (data not shown). The single strain with a KatG S315T alteration is positioned in a completely separate branch and comprises the only Beijing strain found. From this we conclude that strain MTB12 is a foreign strain imported into the Lisbon area, not being part of the endemic circulating MDR clones. Comparison of the 12 loci MIRU with the SITVITWEB showed that MTB12 possess the MIRU international type MIT83, which corresponds to a modern Beijing type.

Given the high-level ethionamide resistance observed in our isolates, the inclusion of ethionamide in second-line treatment regimens will have no benefit and other therapeutic combinations must be considered.[16] Our data point to the need for rapid diagnostic methods for the detection of these strains and strong laboratory support to provide timely and accurate drug resistance information to guide the implementation of appropriate therapy.[4] While our study is limited by the size of the sample evaluated, the strains investigated account for ~45% of all the MDR-TB strains isolated in Lisbon during this period.[17–19]

In conclusion, we report that the presence of a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region is associated with high-level resistance to both isoniazid and ethionamide among the MDR-TB strains circulating in Lisbon. Furthermore, we demonstrate that MDR-TB cases in Lisbon continue to be caused by a closely related family of strains, identified several years ago as being associated with MDR/XDR-TB.[20] This mutational pattern also suggests the involvement of strain-specific factors that could be a feature of Portuguese MDR-TB strains with relevance for their appropriate treatment.

Acknowledgements

We are thankful to Emmanuelle Cambau for fruitful discussions during the European qDST Study Meetings and to Ricardo Parreira for help with the phylogenetic analysis. The Grupo de Micobactérias of the Instituto de Higiene e Medicina Tropical/UNL is grateful to Becton Dickinson (USA) and Quilaban (Portugal) for having provided the TB-eXIST module.

Funding

This work was supported by grant EU-QREN/COMPETE-PTDC/SAU-FCF/102807/2008 from Fundação para a Ciência e a Tecnologia (FCT; Portugal) and Project Ref[a] SDH49: 'Early Molecular Detection of M/XDRTB in the Great Lisbon Healthcare Region' from Fundação Calouste Gulbenkian (FCG; Portugal). D. M. and J. P. were supported by grants SFRH/BD/65060/2009 and SFRH/BD/45388/2008, respectively, from FCT.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Hazbón M, Brimacombe M, del Valle M *et al*. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2006; **50**: 2640–9.

2 Vilchèze C, Wang F, Arai M *et al*. Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat Med* 2006; **12**: 1027–9.

3 Morlock G, Metchock B, Sikes D *et al*. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* 2003; **47**: 3799–805.

4 Viveiros M, Martins M, Couto I *et al*. Molecular tools for rapid identification and novel effective therapy against MDRTB/XDRTB infections. *Expert Rev Anti Infect Ther* 2010; **8**: 465–80.

5 Perdigão J, Macedo R, João I *et al*. Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. *Microb Drug Resist* 2008; **14**: 133–43.

6 Springer B, Lucke K, Calligaris-Maibach R *et al*. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. *J Clin Microbiol* 2009; **47**: 1773–80.

7 Machado D, Couto I, Perdigão J *et al*. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS ONE* 2012; **7**: e34538.

8 Supply P, Allix C, Lesjean S *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit–variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; **44**: 4498–510.

9 Allix-Béguec C, Harmsen D, Weniger T *et al*. Evaluation and strategy for use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* 2008; **46**: 2692–9.

10 Demay C, Liens B, Burguiere T *et al*. SITVITWEB–a publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. *Infect Genet Evol* 2012; **12**: 755–66.

11 Brossier F, Veziris N, Truffot-Pernot C *et al*. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011; **55**: 355–60.

12 Leung E, Ho P, Yuen K *et al*. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. *Antimicrob Agents Chemother* 2006; **50**: 1075–8.

13 Hazbón M, Motiwala AS, Cavatore M *et al*. Convergent evolutionary analysis identifies significant mutations in drug resistance targets of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2008; **52**: 3369–76.

14 David S, Duarte EL, Leite CF *et al*. Implication of the RD^{Rio} *Mycobacterium tuberculosis* sublineage in multidrug resistant tuberculosis in Portugal. *Infect Genet Evol* 2012; **12**: 1362–7.

15 Fenner L, Egger M, Bodmer T *et al*. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2012; **56**: 3047–53.

16 Falzon D, Jaramillo E, Schünemann H *et al*. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. *Eur Respir J* 2011; **38**: 516–28.

17 Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. *Ponto da Situação Epidemiológica e de Desempenho de 2011. Relatório para o Dia Mundial da Tuberculose, avaliação preliminar em Março 2012*. Direcção-Geral da Saúde, Lisboa, Portugal, 2012.

18 Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. *Ponto da Situação Epidemiológica e de Desempenho de 2010. Relatório para o Dia Mundial da Tuberculose, avaliação preliminar em Março 2011*. Direcção-Geral da Saúde, Lisboa, Portugal, 2011.

19 Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. *Ponto da Situação Epidemiológica e de Desempenho de 2009. Relatório para o Dia Mundial da Tuberculose, avaliação preliminar em Março 2010*. Direcção-Geral da Saúde, Lisboa, Portugal, 2010.

20 Portugal I, Covas MJ, Brum L *et al*. Outbreak of multiple drug-resistant tuberculosis in Lisbon: detection by restriction fragment length polymorphism analysis. *Int J Tuberc Lung Dis* 1999; **3**: 207–13.